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Plant Physiology and Biochemistry 216 (2024) 109144

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

The *Lotus japonicus NPF4.6* gene, encoding for a dual nitrate and ABA transporter, plays a role in the lateral root elongation process and is not involved in the N_2-fixing nodule development

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ARTICLE INFO

Handling editor: Mario De Tullio

Keywords:
Lotus japonicus
Nitrate
Abscisic acid
Transport
Root phenotyping

ABSTRACT

Plant root development depends on signaling pathways responding to external and internal signals. In this study we demonstrate the involvement of the *Lotus japonicus LjNPF4.6* gene in the ABA and nitrate root responding pathways. *LjNPF4.6* expression in roots is induced by external application of both nitrate and ABA. *LjNPF4.6* promoter activity is spatially localized in epidermal cell layer and vascular bundle structures with the latter pattern being controlled by externally applied ABA. *LjNPF4.*6 cRNA injection achieves both nitrate and ABA uptake in *Xenopus laevis* oocytes and the analyses of *L. japonicus* knock-out insertion mutants confirmed the role played by LjNPF4.6 in root nitrate uptake. The phenotypic characterization of the *Ljnpf4.6* plants indicates the role played by *LjNPF4.6* in the root program development in response to exogenously applied nitrate and ABA. Based on the presented data, the mode of action of this transporter is discussed.

1. Introduction

Plants, as sessile organisms, must cope with sudden environmental variations leading to changes of the nutrient availability in the surrounding soil. One of the successful strategies implemented by plants deals with the optimization of nutrient uptake, storage/remobilization as well as distribution into different plant tissues. Nitrogen is the most essential nutrient for plant growth and nitrate (NO_3^-) together with ammonium (NH_4^+) represent the major forms of inorganic nitrogen source for plant growth and metabolism with the former being the largely dominant supply form for most plants in temperate climates soil (Miller and Cramer, 2005). Nitrate is quickly dissolved in the soil solution and its high mobility determines a patchy concentration distribution within the soil. Nitrate also plays a role as signaling molecule involved in the control of many physiological and developmental processes. In particular, nitrate signaling is involved in gene regulation (Wang et al., 2004; Omrane et al., 2009), root and shoot growth (Guo et al., 2005; Remans et al., 2006; Zhang and Forde, 2000; Walch-Liu et al., 2000, 2006; Rahayu et al., 2005), germination (Alboresi et al., 2005), flowering (Castro Marin et al., 2011), to finely adapt plant growth and nutrition with the changing environmental conditions (Krouk et al., 2010).

In angiosperms the low-affinity nitrate transport proteins (LATS) are mainly represented by the Nitrate Transporter 1/Peptide Transporter Family (NPF), which include a large number of genes (53 members in *Arabidopsis thaliana*, 80 in *Oriza sativa*; 86 in *Lotus japonicus*), divided into eight subfamilies (Léran et al., 2014; Sol et al., 2019). To date, nitrate transport activity has been reported for 17 out of 53 NPF proteins in *Arabidopsis thaliana* (Corratgé-Faillie and Lacombe, 2017). Dual affinity of some NPFs have been reported (AtNPF6.3; Liu et al., 1999 and MtNPF6.8; Morére-Le Paven et al., 2011), whereas a high-affinity nitrate transport activity has been associated to the MtNPF1.7 and MtNPF7.6 proteins (Bagchi et al., 2012; Wang et al., 2020). The nitrate uptake from soil, long-distance transport within the plant body and distribution from source to sink tissues is controlled through a network of regulated spatio-temporal pattern of expression of *NPF* genes (Noguero and

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https://doi.org/10.1016/j.plaphy.2024.109144
Received 10 May 2024; Received in revised form 18 September 2024; Accepted 21 September 2024
Available online 23 September 2024

Lacombe, 2016; Wang et al., 2018a). An emerging feature reported for several NPF proteins is the plasticity of their transport capabilities. NPF can transport different substrates other than nitrate such as: chloride, potassium, di/tri-peptides, amino acids, glucosinolates, steroidal glycoalkaloids, monoterpene indole alkaloids, malate, auxin, abscisic acid (ABA), gibberellic acid and jasmonic acid (Frommer et al., 1994; Jeong et al., 2004; Waterworth and Bray, 2006; Krouk et al., 2010; Kanno et al., 2012; Nour-Eldin et al., 2012; Saito et al., 2015; Tal et al., 2016; Kanstrup and Nour-Eldin, 2022). In particular, the capability to transport phytohormones might suggest the involvement of NPF proteins in mechanisms of responsiveness controlled by synthesis, transport, perception and signaling of endogenous hormones. The involvement of *NPF* transporters in regulatory cross-talks linking different signals is also suggested by the variety of phenotypes reported for *npf* mutants (Krouk et al., 2010; Kanno et al., 2012; Saito et al., 2015; Chiba et al., 2015; Tal et al., 2016). A paradigmatic example of a *NPF* member involved in the NO_3^-/hormones crosstalk is represented by the nitrate transceptor *AtNPF6.*3 that functions as a nitrate sensor and transporter in *A. thaliana* (Ho et al., 2009). *AtNPF6.3* was found to be a NO_3^--controlled auxin transporter, responsible for the basipetal flux of auxin in the lateral root primordium in low NO_3^- conditions. Inversely, NO_3^- provision represses the auxin transport activity of *AtNPF6.3* and provokes auxin accumulation in the primordium with consequent lateral root elongation (Ho et al., 2009; Krouk et al., 2010; Bouguyon et al., 2015). On the other side, the NO_3^-/ABA cross-talk mechanisms involved in the control of the root architecture are less clear. ABA, commonly known as "stress hormone" may be quickly accumulated in plants in response to abiotic stress conditions such as drought and salt. When the environment is optimal, ABA is reduced to basal levels which promote optimal growth. Modulation of ABA levels in tissues and cells is controlled by synthesis/degradation, metabolism, (de)conjugation and transport processes (Leung and Giraudat, 1998; Lin et al., 2007; Gonzalez-Guzman et al., 2012). ABA can act as both positive and negative signal and a positive role in many plant development programs has been reported (Koornneef et al., 1984). ABA is involved in the development of root architecture via the control of several steps such as lateral root initiation, meristem activation and elongation (De Smet et al., 2003; Ding and De Smet, 2013; Duan et al., 2013). In some of these processes a link with nitrate external conditions has been reported. As an example, in *A. thaliana* high NO_3^- external concentrations (50 mM) are repressive for root branching and this phenotype is dependent by *ABA1*, *ABA2* and *ABA3*, three genes involved in ABA biosynthesis (Signora et al., 2001). The inhibitory action of nitrate is counteracted in *aba1-3* mutant genotypes (Signora et al., 2001). The positive role of ABA on root development has been associated with an action on meristem maintenance in *Arabidopsis* (Zhang et al., 2010; Talboys et al., 2011). Ondzighi-Assoume et al. (2016) demonstrated that ABA accumulation in the root endodermis, controlled by nitrate external concentrations, represents a key positive signal for root elongation. This phenomenon could not be explained by an increase in ABA synthesis (*aba* mutants having no effects), but rather by the release of bioactive ABA from the inactive storage form ABA-glucose ester (ABA-GE) via the action of the *β-GLUCOSIDASE 1* (*BG1*) (Ondzighi-Assoume et al., 2016). Nitrate and ABA are also involved in the control of the nodulation process that takes place on the roots of legumes as result of the symbiotic interaction with rhizobia (Ding and Oldroyd, 2008). Application of exogenous ABA inhibits the signaling pathways leading to nodule formation, bacterial infection, gene expression and N_2-fixation (Suzuki et al., 2004; Tominaga et al., 2009). Nitrate is known to act as nutrient and signal in the control of all the different steps of nodulation during the Symbiotic Nitrogen Fixation (SNF; Omrane and Chiurazzi, 2009; Valkov et al., 2020). Furthermore, some analogies and physiological relationships between nodule and lateral root organogenesis programs can be drawn (Hirsch, 1992) making a possible link between ABA and nitrate in the control of the nodulation process, worth to be investigated.

ABA exists naturally in plants as both an anionic form (ABA^-) and a protonated form (ABAH). ABAH can diffuse passively through the plasma membrane, and the diffusion declines with alkalization of the cytoplasm, which increases during osmotic stresses (Karuppanapandian et al., 2017). ABA is also actively transported between the cells and so far, four types of transporters have been identified in plants (Léran et al., 2020). The first one includes a subgroup of the ATP BINDING CASSETTE (ABC) transporters, namely AtABCG25, 30, 31 and 40 acting in the delivery of ABA from the endosperm to the embryo (Kang et al., 2010). An ABCG20 transporter has been also identified in *M. truncatula* that it is involved in root development and seed germination (Pawela et al., 2019). The second type is a Detoxification Efflux Carrier/Multidrug and Toxic Compound Extrusion (DTX/MATE) identified in *A. thaliana* (AtDTX50) as an efflux transporter playing a role in ABA sensitivity and drought tolerance (Zhang et al., 2014). The third type is represented by a PLASMA MEMBRANE PROTEIN1 of the ABA-induced Wheat Plasma Membrane Polypeptide (AWPM) family involved in ABA influx in *Oriza sativa* (OsPM1) and controlling drought response (Yao et al., 2018). However, the most numerous type of ABA transporters are members of the NPF family identified up to now in *A. thaliana*, *M. truncatula* and *S. lycopersicum* (Kanno et al., 2012; Pellizzaro et al., 2014; Corratgè-Faillie and Lacombe, 2017; Shohat et al., 2020). In *A. thaliana* most of the ABA transporters functionally characterized are members of the clade NPF4 although contrasting results were obtained for a few of them in different heterologous expression systems (Kanno et al., 2012; Chiba et al., 2015; Léran et al., 2020). However, an univocal correspondence between ABA transport capacity and subgroup 4 of the NPF family cannot be established (Chiba et al., 2015). The diffused capability of the NPF to transport ABA, which in some cases is coupled to a nitrate transport activity draws an intriguing resemblance to the case of *AtNPF6.3* (Krouk et al., 2010). However, the interaction between the nitrate and ABA substrates has been tested in the case of AtNPF4.6 and no evident interaction between the two transport activities has been found (Kanno et al., 2013). More recently, a post-translational regulation via phosphorylation of AtNPF4.6 has been reported controlling protein stability and ABA import activity (Zhang et al., 2021).

Here we report the functional characterization of *LjNPF4.6* aimed to investigate its role in the control of root and nodulation developmental programs. The characterization of three independent null mutants allowed to draw a possible role of *LjNPF4.6* in the cross-talk between nitrate and ABA signaling pathways controlling the lateral root elongation program in *L. japonicus*.

2. Material and methods

2.1. Plant material and growth conditions

All experiments were carried out with *Lotus japonicus* ecotype B-129 F12 GIFU (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1997). Plants were cultivated in a growth chamber with a light intensity of 200 μmol $m^{-2}.sec^{-1}$ at 23 °C with a 16 h/8 h day/night cycle. Phenotypic characterizations have been performed as described by Rogato et al. (2022). Solid growth media had the same composition as that of Gamborg B5 medium (1970), except that $(NH_4)_2SO_4$ and KNO_3 were omitted and substituted by the proper N source at the required concentration. KCl is added, when necessary to the medium to replace the same concentrations of potassium source. The media containing vitamins (Duchefa catalogue G0415) are buffered with 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES; Duchefa catalogue M1503.0250) and pH adjusted to 5.7 with KOH. After germination, unsynchronized seedlings were discarded.

M. loti inoculation was performed as described by Barbulova et al. (2005). The root system was shielded from light access (even overhead light) as described in Rogato et al. (2021). The strain R7A was used for the inoculation experiments, grown in liquid TYR-medium supplemented with rifampicin (20 mg/L).

Root and shoot length are measured with ImageJ software

(Schneider et al., 2012).

2.2. L. japonicus transformation procedures

Binary vectors were electroporated in the *Agrobacterium rhizogenes* 15834 strain (White and Nester, 1980). *A. rhizogenes*-mediated *L. japonicus* transformation has been performed as described in D'Apuzzo et al. (2015). Inoculation of composite plants is described in Santi et al. (2003).

2.3. Constructs preparation

The *pLjNPF4.6*-GUS T-DNA construct containing 433 bp upstream of the ATG codon was prepared in the following way: PCR amplified fragment have been obtained on genomic DNA with the specific forward oligonucleotide containing a *Sal*I site in combination with the reverse primer containing the *Bam*HI site (Table S1). The amplicon then subcloned as *Sal*I-*Bam*HI fragments into the pBI101.1 vector (Jefferson, 1987) to obtain the translational fusion plasmid.

The plasmid for expression in *Xenopus laevis* oocytes was prepared in the following way: cDNA prepared from root RNA amplified with the forward primer containing the *Xba*I site in combination with the reverse primer containing the *Hind*III site (Table S1). The *Xba*I/*Hind*III double digested 1920 bp fragment was subcloned into the pGEMHE plasmid (containing the 5′-UTR and 3′-UTR of the *Xenopus laevis* β-*GLOBIN* gene; Liman et al., 1992), pre-digested *Xba*I-*Hind*III to obtain pGEMHE4.6. The correct coding sequence of *LjNPF4.6* has been verified by sequencing.

2.4. Functional analysis of LjNPF4.6 in Xenopus laevis oocytes

pGEMHE4.6 was linearized with *Nhe*I and capped mRNA transcribed *in vitro* using the mMessage mMachine T7-ultra Kit (Life Technologies). Oocytes preparation is described in (Lacombe and Thibaud, 1998). Oocytes obtained surgically from anesthetized *Xenopus* were defolliculated by a 1 h collagenase treatment (1 mg.ml−1, type IA, Sigma Chemical, Saint-Louis, MO) in a medium containing (in mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES-NaOH (pH 7.4). Stage V and VI oocytes were selected and placed in a ND96-modified medium containing in mM: 2 KCl, 96 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 2.5 sodium pyruvate, pH 7.5, supplemented with gentamicin sulfate (50 mg/mL-1). Defolliculated oocytes were injected with 20 ng of complementary RNA (cRNA) and stored in the modified ND96 medium described above. Two days after injection, batches of 10 injected oocytes were incubated in 1 mL of ND96 solution at pH 5.5 or 7.5 supplemented with 30 mM $^{15}NO_3$ supplied as $K^{15}NO_3$ for 2 h at 18 °C. Oocytes were then rinsed five times in 15 mL cold modified ND96 solution. Oocytes were then analyzed for total N content and atom % ^{15}N abundance by Continuous-Flow Mass Spectrometry, using a PyroCube Eurovector elementar analyzer coupled with an IsoPrime mass spectrometer (Elementar, Germany). Oocytes injected with *AtNPF6.*3 cRNA and water were used as positive and negative controls, respectively. Results are expressed in pmol.oocyte-1.min-1.

For ABA uptake, oocytes were incubated for 20 min in 1 ml of ND96 containing the indicated concentration of ABA solution (^{3}H-ABA, American Radiolabelled Chemicals and 90% of cold ABA, Sigma). Oocytes were then washed 4 times in 15 ml of ND96 solution (4 °C) containing 5 μM of cold-ABA. Each oocyte was then dissolved in 100 μl of 2% Sodium Dodecyl Sulfate (SDS). Lysis solution was then mixed to 3 ml of scintillating solution (ULTIMAGOLD, PerkinElmer). Incorporated radioactivity was measured by Liquid-Scintillation analyzer (Tri-Carb 2100 TR, PerkinElmer).

2.5. Quantitative Real-time RT-PCR

Real time PCR is performed with a CFX96 Touch Real-time PCR Detection System (Biorad) and Biorad CFX Maestro software. SYBR was used to monitor dsDNA synthesis. The procedure is described in Moscatiello et al. (2018). The ubiquitin (*UBI*) gene (AW719589) was

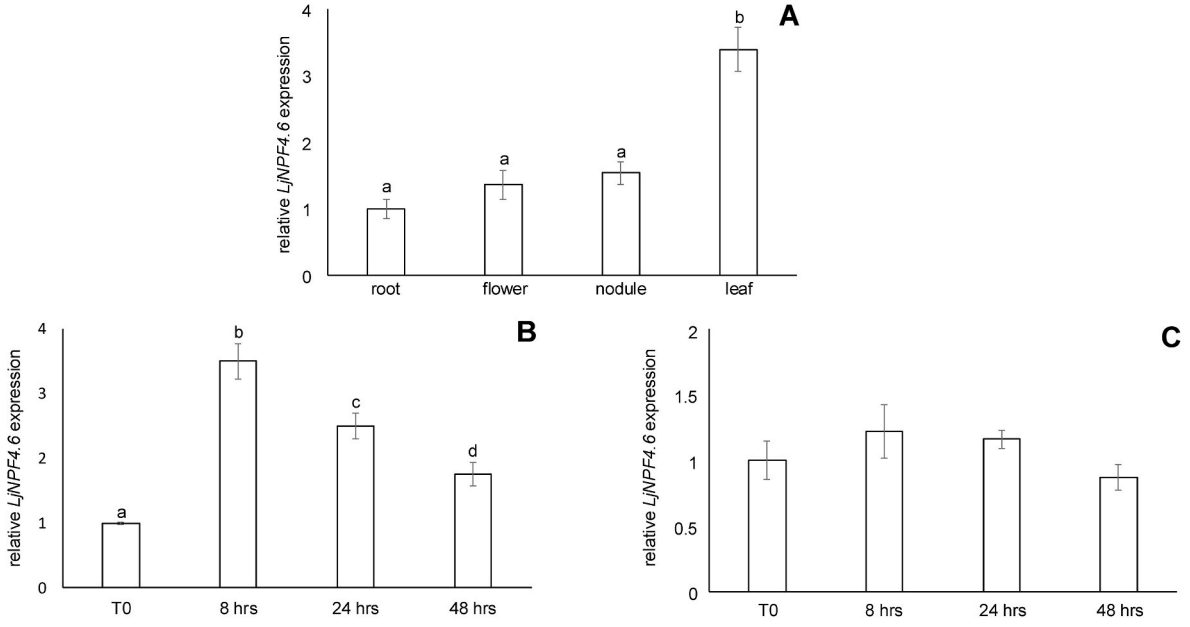

Fig. 1. *LjNPF4.6* transcriptional profiles. A, *LjNPF4.6* relative expression in different organs. RNAs were extracted from wild type plants grown on B5/2 Gamborg derivative medium with 1 mM KNO_3 as N source, at three weeks after *M. loti* inoculation. Mature flowers were obtained from *Lotus* plants grown in the growth chamber. Expression levels are normalized with respect to the internal control *UBI* gene and plotted relative to the expression in root. B,C, Time course of the *LjNPF4.6* transcriptional response in roots (B) and leaves (C) of wild type *L. japonicus* plants after transfer from Gln 1 mM to KNO_3 5 mM conditions. Times of RNA extractions (hrs) are indicated in the figure. Expression levels are normalized with respect to the internal control *UBI* gene. The *LjNPF4.6* expression in roots and leaves did not change at 8, 24 and 48 h from the transfer and hence, the level of expression was plotted relative to the T0 in B and C. Data bars represent the mean and standard deviations of data obtained with RNAs extracted from three different sets of plants and 3 RT-qPCR experiments. Different letters above bars indicate significant differences among samples ($p < 0.01$, One-way ANOVA).

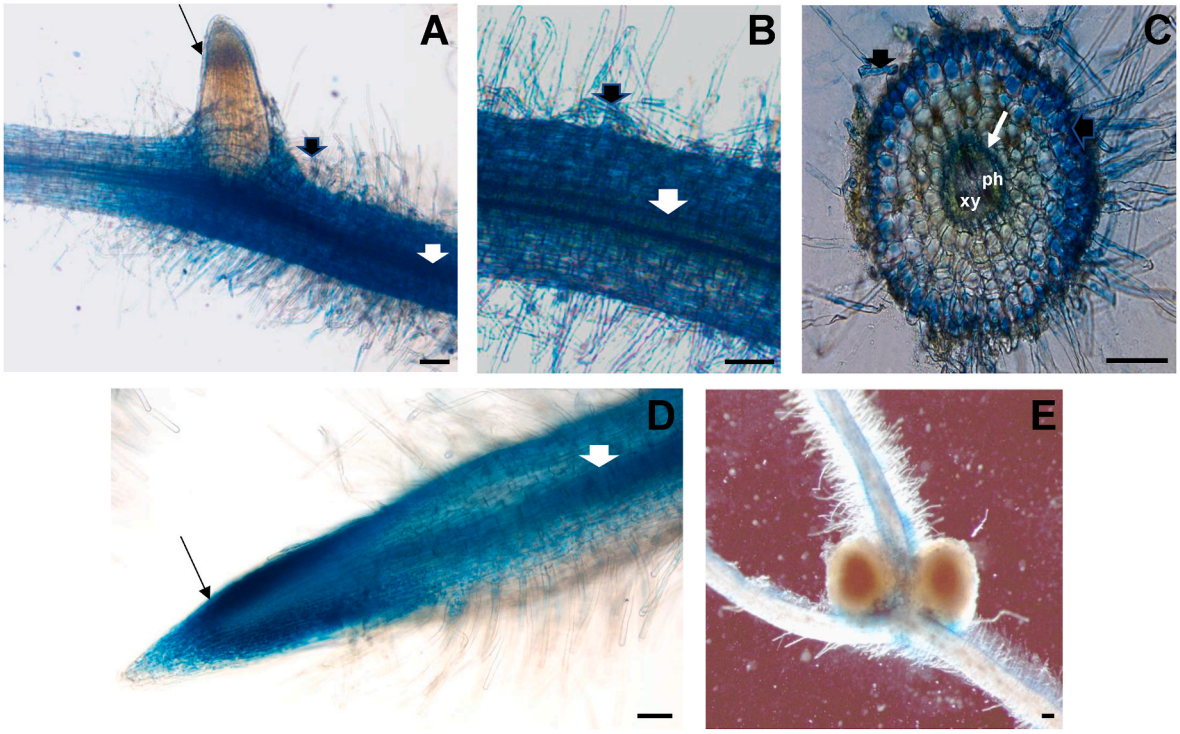

Fig. 2. Representative spatial profiles of the *LjNPF4.6* promoter activity in transgenic hairy roots. A,B,D,E, GUS activity in whole-mount, stained hairy roots. C, cross section of a stained hairy root. E, GUS activity is not detected within nodule tissues. Black arrows indicate the blue staining detected in lateral root cap and columella cells (panels A,D). White arrow-heads indicate the blue staining detected in vascular stele (panels A–C). Black arrow-heads indicate blue staining detected in epidermis and root hairs (panels A,B,C). White arrow indicates the blue staining detected in the endodermis/pericycle layers of the central stele (panel C). ph = phloem; xy = xylem. Bar on the left = 50 μm.

used as an internal standard. The oligonucleotides used for the qRT-PCR are listed in the Supplementary Table S1.

2.6. LORE1 lines analyses

LORE1 lines 30000742, 30081602 and 30087442 were obtained from the LORE1 collection (Urbanski et al., 2012; Malolepszy et al., 2016). The plants in the segregating populations were genotyped and *LjNPF4.6* expression in homozygous plants tested with primers listed in Table S1. After PCR genotyping, shoot cuts of the homozygous plants were cultured in axenic conditions was performed as described in Vittozzi et al. (2021).

2.7. Histochemical GUS analysis

Histochemical staining of whole plant and sections material were performed as described by Rogato et al. (2008, 2016).

2.8. Root nitrate influx

Root nitrate influx assay was performed as previously described by Munos et al. (2004). Lotus plants were grown for 12 days on vertical agar plates containing Gamborg B5 derivative medium supplemented with 1 mM glutamine as sole N source. Plants were transferred to 0.1 mM $CaSO_4$ solution for 1 min, then to nutrient solution (pH5.7) containing 5 mM $^{15}NO_3^-$ (99% atom excess ^{15}N) for 5 min and finally washed in 0.1 mM $CaSO_4$ for 1 min. Roots were then separated from shoots and dried at 70 °C for at least 48 h. After determination of dry weight, the samples were analyzed for total nitrogen and atom % ^{15}N using a PyroCube elementar analyzer coupled with an IsoPrime mass spectrometer (Elementar, Germany).

2.9. Statistical analysis

Statistical analyses were performed using the VassarStats two-way factorial ANOVA for independent samples program or Chi-Square test of association (http://vassarstats.net/).

3. Results

3.1. LjNPF4.6 displays a nitrate-induced root profile of expression localized in the root epidermis and periphery of the root stele

We have previously reported the complete list of *L. japonicus* NPF members and relative nomenclature (Criscuolo et al., 2012; Valkov and Chiurazzi, 2014; Sol et al., 2019), based on the parameters described in Léran et al. (2014). *LjNPF4.6* corresponds to the LotjaGi2g1v0308900.1 and Lj2g3v1339020.1 identical sequences in the *L. japonicus* Gifu and MG20 accessions, respectively (https://lotus.au.dk; http://www.kazusa.or.jp/lotus; Sato et al., 2008; Mun et al., 2016). *LjNPF4.6* is predicted to encode for a 627 amino acid protein (67.98 kDa predicted mass) containing 12 transmembrane domains with a long amino acid cytoplasmic loop between 6th and 7th TM domains (Fig. S1). A BLAST search against the TAIR database (https://www.arabidopsis.org) indicated that LjNPF4.6 shares the highest value of amino acid identity with AtNPF4.6 (72%), with which also shares the gene structure constituted by five exons and a large 3rd intron. The serine residue 292 of AtNRT1.2/NFF4.6, which is phosphorylated through the action of the C-terminally encoded peptide receptor 2 (CEPR2), involved in the transporter stability and ABA import/response, was also identified (Fig. S1; Zhang et al., 2021).

The qRT-PCR analysis performed with RNAs extracted from different organs of *L. japonicus* indicated a significant ubiquitous expression of *LjNPF4.6* with the highest level observed in leaf tissues (Fig. 1A). This transcriptional profile is consistent with the results reported in the

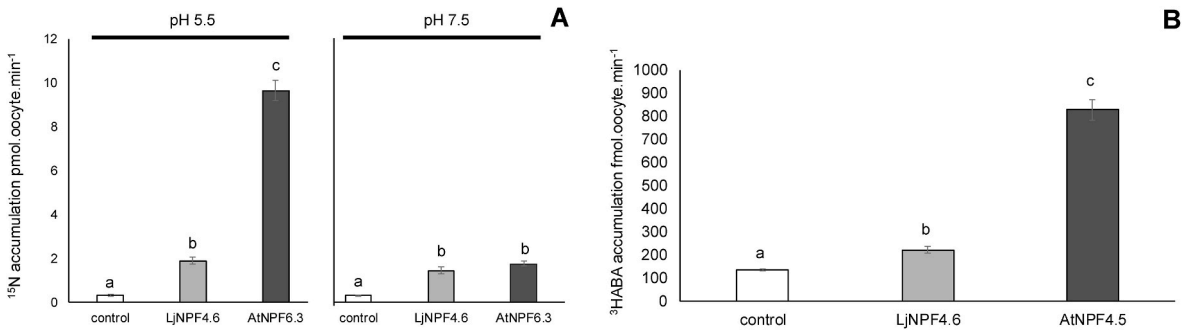

Fig. 3. LjNPF4.6 is a nitrate and ABA importer. A, Control (white bars), *LjNPF4.6*- (grey bars) and *AtNPF6.3* (black bars)-expressing oocytes were incubated with 30 mM ^{15}N-labeled nitrate. The ^{14}N/^{15}N ratio was determined in dried oocytes. Data are mean and SEM (n = 6–12 oocytes). B, ABA accumulation in *LjNPF4.6* and *AtNPF4.5* expressing oocytes incubated with 1 μM ^{3}HABA at pH 5.5. Different letters above bars indicate significant differences among samples ($p < 0.01$, one-way ANOVA).

L. japonicus expression atlas (https://lotus.au.dk/expat/; Mun et al., 2016). The comparison of the relative levels of expression of the 10 genes identified within the clade 4 of the *LjNPF* family (Sol et al., 2019), confirmed the higher expression of *LjNPF4.6* in both root and leaves as compared to the other *LjNPF4* genes (Fig. S2). High concentrations of nitrate (5 mM) induced a rapid accumulation of *LjNPF4.6* mRNA in roots (3.5-fold) but not in leaves in the time course experiments shown in Fig. 1B and C. The level of expression in roots reached a peak at 8 h from the transfer to progressively decrease at 24 and 48 h (Fig. 1B).

To obtain information about the spatial expression profile of *LjNPF4.6* in the root tissue, we have analyzed *L. japonicus* hairy roots transformed with a prom-*LjNPF4.6-gus*A fusion. Composite plants were cultured on B5/2 Gamborg medium (12 mM KNO_3) to allow elongation of the transgenic roots. GUS activity was detected in the epidermal cell layer and root hairs, columella and lateral root cap cells and vascular bundle structures of both mature and young roots (Fig. 2A,B,D). The root cross section shown in Fig. 2C allowed to localize the vascular GUS staining at the periphery of the root stele (endodermis/pericycle cell layers). GUS activity was not detected in the nodule tissues (Fig. 2E).

3.2. *LjNPF4.6 transports KNO_3 and ABA in Xenopus laevis oocytes*

The nitrate-dependent *LjNPF4.6* expression observed in *Lotus* wild type roots (Fig. 1B) prompted us to investigate whether LjNPF4.6 is capable to transport nitrate in a heterologous experimental system. *Xenopus* oocytes were injected with *in vitro*-synthesized *LjNPF4.6* complementary RNA (cRNA) for functional assays. Two days after the injections, oocytes have been tested for $^{15}NO_3$ uptake activity at a 30 mM concentration at two different pH values (5.5 and 7.5) and the *AtNPF6.3* injected oocytes were used as positive experimental control. A significant increase of $^{15}NO_3$ accumulation as compared to water injected controls has been scored in both batches of NPF-injected oocytes (83% for the *LjNPF4.6*; Fig. 3A). The *AtNPF6.3*-dependent $^{15}NO_3$ uptake activity was strongly affected by pH, whereas the one induced by *LjNPF4.6* was not sensitive to pH (Fig. 3A).

Furthermore, as a *bona-fide* ABA transport activity has been reported for four out of the seven members of the NPF4 subfamily in *A. thaliana* (Léran et al., 2020), we decided to test whether *LjNPF4.6* is also capable to trigger ABA accumulation in injected *Xenopus* oocytes. In this case the positive control was represented by the *AtNPF4.5* injected oocytes. ^{3}H-labeled ABA was quantified in oocytes at pH 5.5 after 20 min incubation. *LjNPF4.6*-injected oocytes accumulated significant increased amount of ^{3}H (40%) as compared to water-injected control indicating that ABA is a substrate for this transporter (Fig. 3B).

3.3. *Phenotypic characterization of LjNPF4.6 LORE1-insertion null mutants*

The functional characterization of the *LjNPF4.6* gene was based on the isolation of three independent insertion mutants isolated from the *L. japonicus* LORE1 insertion collection obtained in the GIFU ecotype (Urbanski et al., 2012; Malolepszy et al., 2016). Lines 30000742, 30081602 and 30087442, bearing retrotransposon insertions in the fourth and fifth exon (Fig. S3), were genotyped by PCR. Shoot cuttings of plants homozygous for the insertion event into the *LjNPF4.6* gene were cultured in axenic conditions and transferred, after root induction, to the growth chamber for seeds production. End-point reverse transcription-PCR analyses revealed no detectable *LjNPF4.6* mRNA in leaf tissues of the three homozygous lines 30000742, 30081602 and 30087442, hereafter called *Ljnpf4.6-1, Ljnpf4.6-2* and *Ljnpf4.6-3*, respectively.

As first phenotypic characterization of the *Ljnpf4.6* plants we have tested whether the $^{15}NO_3^-$ transport revealed in *Xenopus laevis* oocyte was physiologically relevant *in planta* by analyzing the effect of the *LjNPF4.6* gene knockout on root nitrate influx. Plants were grown for 12 days on B5 derived medium without nitrate source (but with 1 mM

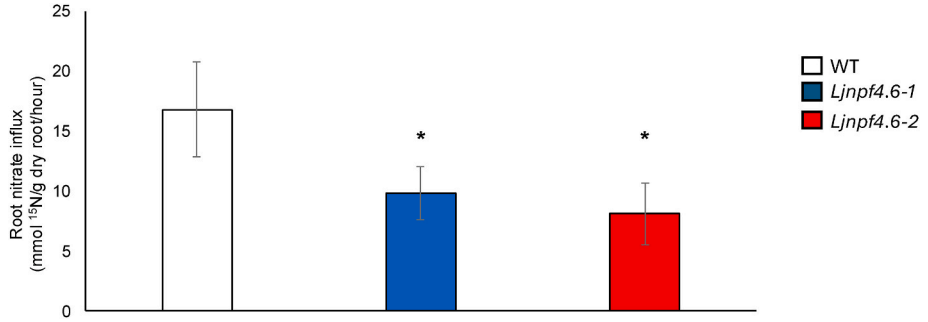

Fig. 4. Nitrate influx in *L. japonicus* roots. ^{15}N content is quantified in the indicated genotypes in short-term (5 min) influx experiments (n = 5 batches of 2/3 seedlings). Values are means ± SE. Asterisks indicate significant differences among samples after comparison with wild type roots (two-sided *t*-test, $p < 0.05$).

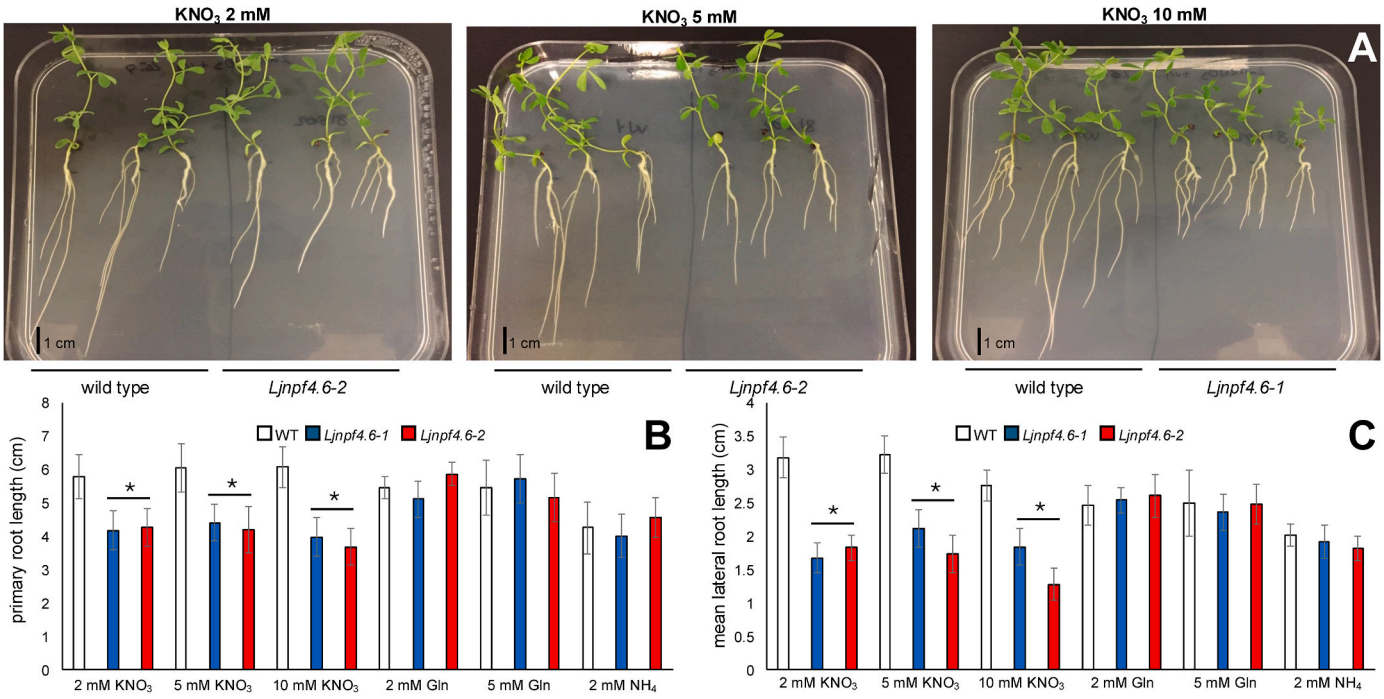

Fig. 5. Root phenotypes of wild type and *ljnpf4.6* mutants grown in the presence of different N sources and concentrations. A, representative images of significant root phenotypes observed in the presence of different KNO_3 concentrations. B, primary roots length. C, mean of lateral roots length. Length of at least 0.25 cm long lateral roots were scored 3 weeks after sowing. Plant genotypes, potassium nitrate, glutamine and ammonium succinate concentrations are indicated. Data bars represent the mean and standard errors obtained from 3 independent experiments (12 plants per experiment). Analysis of variance of root length between wild type and *Ljnpf4.6* mutants was conducted with One-way ANOVA; *$p < 0.01$.

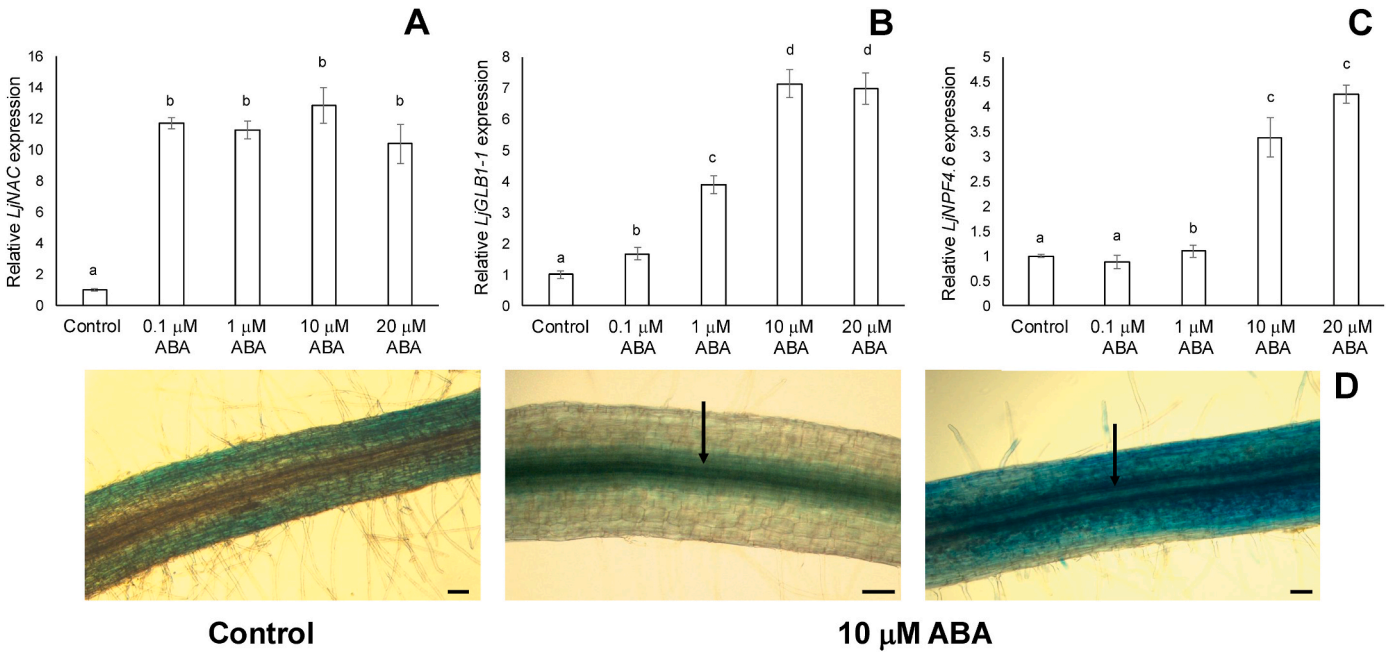

Fig. 6. ABA-dependent profiles of *LjNPF4.6* expression in roots. A, *LjNAC* relative expression. B, *LjGLB1-1* relative expression. C, *LjNPF4.6* relative expression. RNAs were extracted from roots of wild type and *Ljnpf3.1-1* plants grown for 10 days on B5/2 derived medium with 10 mM KNO_3 as sole N source and then transferred for 24 h on the same medium supplied with ABA at the indicated concentrations. Plant genotypes are indicated. Data bars represent the mean and standard deviations of data obtained with RNAs extracted from three different sets of plants and 3 RT-qPCR experiments. Different letters above bars indicate significant differences among samples ($p < 0.01$, One-way ANOVA). **D,** Representative images of the GUS profile of expression in hairy roots transformed with the p*LjNPF4.6*-GUS fusion with/out ABA treatment. Composite plants with elongated hairy roots were transferred on medium without N sources for 7 days and then transferred on the same medium with/out 10 µm ABA. Arrows indicate the staining in the central stele. Bars on the right = 50 µm ABA treated roots: n = 38; p = 0.045. Untreated roots: n = 38; p = 0.04. Chi-Square test of association.

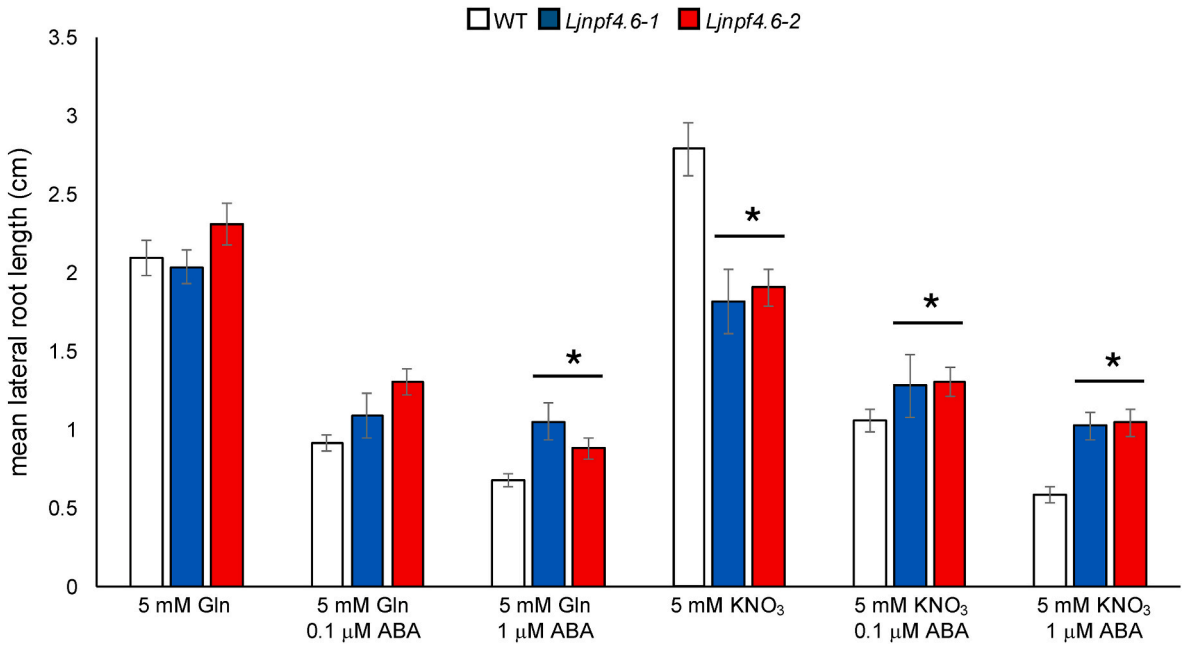

Fig. 7. Effects of ABA on lateral root length of wild type and *Ljnpf4.6* plants. One week after sowing, seedlings grown in the presence of Gln 5 mM or KNO_3 5 mM were transferred on the same media supplemented with different concentrations of ABA. Lateral roots length were scored two weeks later. ABA concentrations are indicated. Data bars represent the mean and standard errors obtained from 3 independent experiments (10 plants per experiment). Analysis of variance of lateral root length between wild type and *Ljnpf4.6* mutants was conducted with One-way ANOVA; *$p < 0.01$.

glutamine as the sole N source) and exposed to 5 mM $^{15}NO_3^-$ for a short-term acquisition assay (5 min). The comparison between nitrate uptake in wt and *Ljnpf4.6* mutants showed a significant impairment in $^{15}NO_3^-$ influx (50%) into the roots of knockout plants (Fig. 4).

In order to verify whether the nitrate-dependent profile of expression observed in roots (Fig. 1B) as well as the involvement in the root nitrate influx (Fig. 4) could be associated to specific roles of *LjNPF4.6* in nitrate responsive root developmental pathways, we have compared different growth phenotypic traits of wild type and *Ljnpf4.6* plants incubated in the presence of different KNO_3 concentrations as sole N source. A clear-cut Short Root (SR) phenotype was revealed in both *Ljnpf4.6-1* and *Ljnpf4.6-2* allelic KO mutants with primary and lateral root length significantly reduced as compared to wild type plants (Fig. 5A). The quantification of the root length parameters indicated similar phenotypes in the whole range of tested KNO_3 concentrations, with a significant decrease of the primary root length in the two *Ljnpf4.6* plants ranging from 25 to 40% as compared to wild type (Fig. 5B). The reduction of the mean lateral root length was even more severe in the *Ljnpf4.6* plants ranging from 33 to 54% as compared to the ones scored in wild type plants (Fig. 5C). However, when we analyzed the root phenotype in the presence of different N sources than nitrate (2 mM and 5 mM glutamine and 2 mM ammonium), we did not score the SR phenotypes indicating a specific nitrate dependent root response (Fig. 5B and C). The slight reduction of primary and lateral root length displayed by both wild type and mutant plants in the presence of ammonium succinate as sole N source (Fig. 5B and C) has been already reported in *L. japonicus* plants (Rogato et al., 2010). The shoot growth parameters did not reveal significant differences in the tested conditions between wild type and mutant genetic backgrounds (Fig. S4). Since at the time we performed the phenotypic characterization of the mutants we did not have a sufficient amount of *Ljnpf4.6-3* seeds, this particular line was tested only for the growth response in the presence of 5 and 10 mM KNO_3 and 5 mM glutamine. The nitrate dependent SR phenotype was confirmed also in this third knockout mutant (Fig. S5), indicating a clear-cut cause-effect relationship between the LORE1 insertions in the *LjNPF4.6* gene and the SR nitrate-dependent phenotype.

3.4. ABA dependent profiles of expression of LjNPF4.6

To gain insight into the involvement of *LjNPF4.6* in ABA signaling pathways, we have examined its transcriptional pattern in response to ABA exogenous treatments. *L. japonicus* wild type plants after 10 days growth on B5-derived medium with 10 mM KNO_3 as sole N source were transferred for 24 h on the same medium supplied with ABA at a concentration ranging from 0.1 µM to 20 µM (Fig. 6). The non-symbiotic hemoglobin *LjGLB1-1* was used as a positive control of the experimental conditions (Bustos-Sanmamed et al., 2011), together with the *L. japonicus* orthologue of the *M. sativa* ABA responsive transcriptional factor *MsNAC* (Wang, 2013). Both these genes exhibited an early induction of expression after the ABA supply, with *LjNAC* being stably induced (about 13 fold) in the whole range of concentrations, whereas the *LjGLB1-1* transcript was progressively increased (7 fold; Fig. 6A and B). Interestingly, the ABA treatment also led to a significant increase of the *LjNPF4.6* transcript (4 fold), which was already revealed in the presence of 1 µM ABA (Fig. 6C).

To further investigate the *LjNPF4.6* ABA-responsive pattern, we have used transgenic hairy roots transformed with the prom-*LjNPF4.6-gus*A to discriminate the ABA-dependent spatial pattern within the profile of GUS activity visualized in Fig. 2. To avoid overlapping between nitrate- and ABA-dependent pattern of GUS activity, composite plants were grown on medium without N source for 7 days and moved to the same medium with/out 10 µM ABA. The staining revealed a significant relationship between ABA treatment and the pattern of GUS activity in the vascular bundles (exclusively or in association to epidermis), whereas the untreated roots displayed staining only in the epidermis region (Fig. 6D).

3.5. The LjNPF4.6 disruption counteracts the inhibitory action of exogenous ABA on lateral root elongation

The ABA transport capability displayed by LjNPF4.6 in the *X. laevis* oocyte (Fig. 3) and its ABA responsive transcriptional pattern (Fig. 6) prompted us to investigate whether *LjNPF4.6* could be involved in the ABA-dependent pathways controlling root development. A positive

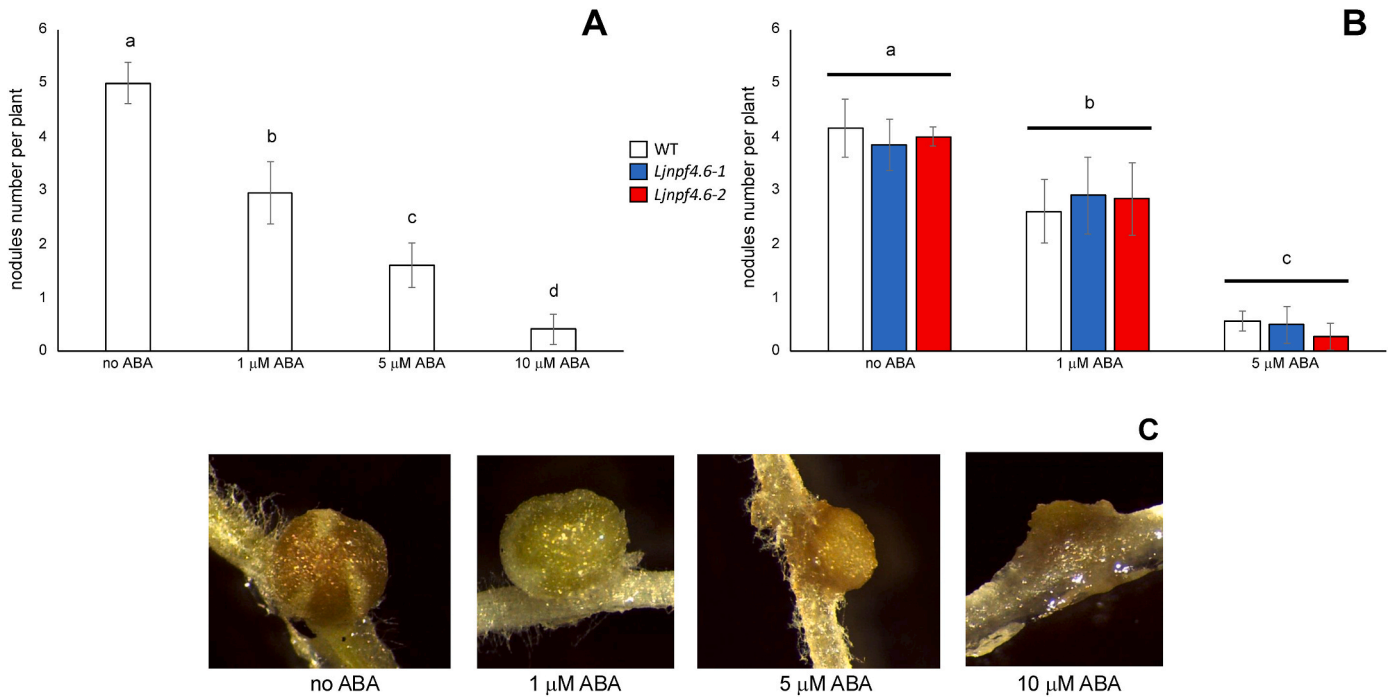

Fig. 8. ABA dependent symbiotic phenotypes of wild type and *Ljnpf4.6* plants. A, nodule number per wild type plants. B, nodule number per wild type and *Ljnpf4.6* plants. Data bars represent the mean and standard errors obtained from 3 independent experiments (12 plants per experiment). Different letters above bars indicate significant differences among samples ($p < 0.01$, One-way ANOVA). C, Representative images of wild type nodules developed in the presence of different ABA concentrations. The different ABA concentrations and plant genotypes are indicated.

correlation between very high NO_3^- provisions (50 mM), repressive for lateral root elongation and the ABA biosynthetic pathways has been demonstrated in *Arabidopsis* adult plants (Signora et al., 2001; Seo and Koshiba, 2002). In *M. truncatula* the *Mtnpf6.8* mutants display a reduced inhibition of primary root elongation in the presence of nitrate and this effect is counteracted by ABA treatment (Pellizzaro et al., 2014). The involvement of *LjNPF4.6* in this nitrate-dependent root elongation repressive pathway cannot be tested in the *Ljnpf4.6* background as we do not observe such an inhibitory effect of high nitrate provision (up to 100 mM) on the root development of *L. japonicus* wild type plants (Fig. S6; Rogato et al., 2010). However, another well described ABA-dependent phenotype is represented by the inhibition of lateral root development observed in the presence of exogenously applied hormone. The ABA-dependent root phenotype in legume plants has been previously described in *M. truncatula*. Liang and Harris (2005), reported a reduction of the primary root length (about 30%) in plants grown in the presence of different ABA concentrations and N regimes (low and high N conditions), as well as an increased lateral root density in the presence of ABA (Liang and Harris, 2005). However, in legume species the root responsiveness to phytohormone treatments cannot be easily predicted as these physiological traits, which have been associated to the acquisition of symbiotic predisposition, appear to be peculiar in this plant family (Liang and Harris, 2005). Therefore, the optimal range of ABA concentrations to highlight clear-cut root phenotypes response had to be tailored for *L. japonicus* wild type plants. Five days old seedlings were transferred on Gamborg B5 medium in the presence of a range of ABA concentrations ranging from 0.1 μM to 10 μM and roots were measured 2 weeks later. The results shown in Fig. S7A indicated a negative effect on the primary root length already in the 0.1–1 μM range of ABA (20% reduction), which was further increased in the presence of 10 μM (39% reduction). However, the most striking phenotype already displayed by *Lotus* plants grown in the presence of 0.1 μM ABA, was the inhibitory effect on lateral root elongation, progressively decreasing from 64% to 90% as compared to control conditions in the tested range of ABA concentrations (Fig. S7B). A slight negative effect of exogenous ABA on the shoot length was observed at 0.1 μM ABA and progressively increased at 10 μM ABA (52% of the lenghts scored in the control conditions; Fig. S7D). We did not reveal any increase of the lateral root density in the presence of ABA (Fig. S7C). Since in our experimental conditions, 0.1 μM ABA was the lowest concentration highlighting a significant change in the mean lateral root length, we selected 0.1 μM and 1 μM as the standard ABA concentrations in all subsequent experiments, to minimize other physiological ABA-dependent effects. In order to uncouple the effects of KNO_3 and ABA we have compared the mean length of lateral roots of wild type and *Ljnpf4.6* plants grown on glutamine or KNO_3 as N source with/out ABA. The specific inhibitory effect of KNO_3 on the *Ljnpf4.6* lateral roots elongation was confirmed as this was not scored in the presence of 5 mM glutamine (Fig. 7). A general ABA-dependent effect was displayed both by wild type and *Ljnpf4.6* plants despite the N regime utilized (Fig. 7). The inhibitory effect of ABA increased at higher concentrations (1 μM) in wild type plants and this was independent by the N regime. Strikingly, the inhibitory effect of both ABA concentrations was counteracted in the mutant background and this was particularly significant in the presence of nitrate. In particular, the mean lateral root length values were reverted as compared to wild type plants (Fig. 7, Fig. S8). This effect was particularly evident at 1 μM ABA. The counteracting action observed in the mutant backgrounds was not revealed for primary root and shoot length phenotypes (Fig. S9). These results indicate the involvement of *LjNPF4.6* in the ABA responsive signaling pathway governing lateral root elongation.

3.6. ABA related symbiotic phenotypes

ABA is known to have an overall inhibitory effect on rhizobia infection, nodule formation and functioning processes (Suzuki et al., 2004; Ding et al., 2008; Tominaga et al., 2009). We have investigated the possible involvement of *LjNPF4.6* on the ABA signaling pathway affecting different steps of the symbiotic interaction by exploiting the range of concentrations previously reported to exert inhibitory effects in

L. japonicus (Suzuki et al., 2004). The progressive inhibitory effect of the exogenously supplied ABA on nodule formation and development in *L. japonicus* wild type plants was confirmed in our experimental conditions with nodulation capability almost completely suppressed at 10 μM ABA (Fig. 8A and B). The comparison of wild type and *Ljnpf4.6* plants did not reveal any difference on the inhibitory responses to exogenous ABA, both in terms of nodule number per plants and nodule development (Fig. 8C) indicating that *LjNPF4.6* does not play a role in the distribution of ABA in response to the *M. loti* inoculation.

4. Discussion

The optimization of root traits is a main focus for sustainability and improvement of production in agricultural systems (Lynch, 2022). The understanding of the physiological mechanisms involved in the control of root development in response to changes of nutrient environmental availability is of crucial importance. Connections between nitrate- and hormone-related signaling pathways controlling plant growth and development have been reported (Signora et al., 2001; Sakakibara et al., 2006; Krouk et al., 2010; Kanno et al., 2012; Chiba et al., 2015; Tal et al., 2016; Poitut et al., 2018). The efficiency of plant responses to changes in external nitrate availability rely on sensing, transport and assimilation systems, which can regulate or be regulated by the endogenous hormonal pathways to link nutrition demands to plant growth and development (Krouk, 2016). In the case of ABA, very little is known about how the biosynthesis or transport of ABA in root tissues may respond to nutritional signals. The reported characterization of the *LjNPF4.6* gene suggests its involvement in the control of lateral root elongation program through a dual role of ABA and nitrate transporter.

The temporal profile of expression shown in Fig. 1 clearly categorizes the *LjNPF4.6* gene as a nitrate-inducible gene with an increase of the transcript abundance detected in roots at 8 h after exposure to nitrate. The spatial pattern of GUS activity triggered by the prom-*LjNPF4.6-gus*A fusion in transgenic hairy roots, with a prominent expression in epidermis, root hair and root tip (Fig. 2) is consistent with that reported for *AtNPF4.6* and *AtNPF6.3,* the two *Arabidopsis* genes mainly involved in nitrate uptake in roots in the low affinity range (Huang et al., 1996, 1999). The results shown in Fig. 6 also indicated a *LjNPF4.6* ABA-responsive profile of expression, which was experimentally validated by checking the up-regulation of two ABA responsive genes. Although we cannot exclude the possibility that the spatial expression of *LjNPF4.6* is controlled by sequences located outside the 5′-UTR region used in the promoter-fusion construct, the pattern of GUS activity detected in the root vascular structures (Fig. 2) nicely correlates to those reported for different ABA transporters (ABC, DTX/MATE, AWPM, NPF; Boursiac et al., 2013). Furthermore, the physiological link between ABA and the spatial pattern of the prom-*LjNPF4.6-gus*A in the root stele has been experimentally confirmed by the data shown in Fig. 6D where the latter is associated to the addition of external 10 μM ABA in plants grown under N-free conditions. ABA is mainly produced in vascular tissues and ABA transporters are likely involved in its efficient distribution from cells where the synthesis takes place to target cells of the ABA action through an export/import mechanism (Kuromori et al., 2018). Recently, the involvement of two members of the *A. thaliana* NPF family, *AtNPF2.12* and *AtNPF2.13* in this root vascular bundle ABA route has been reported (Binenbaum et al., 2023).

Our biochemical characterizations, carried out in the heterologous system *X. laevis* confirmed the involvement of *LjNPF4.6* in nitrate- and ABA-related pathways. We report the capability of LjNPF4.6 to transport NO_3^- and ABA, achieving a significant uptake of these substrates in *Xenopus* oocytes (Fig. 3). The differences in NO_3^- and ABA accumulation observed between LjNPF4.6 and the positive controls (AtNPF6.3 and AtNPF4.5, respectively), have been already reported within the *Arabidopsis* NPF family and are possibly due to differences of expression or activity levels in the *Xenopus* oocytes (Léran et al., 2015, 2020). These results suggest that LjNPF4.6 functions as an NO_3^-/ABA importer, although we cannot exclude the possibility that this protein is a bidirectional transporter as reported for some NPF proteins. The NO_3^- uptake efficiency in oocytes injected with the *LjNPF4.6* was not dependent on pH conditions (5.5–7.5 range, Fig. 3A). The lack of pH effect on the NPF-mediated NO_3^- uptake activity has been reported in other cases in the pH range tested (Hu et al., 2016; Wang et al., 2018b). The partial conservation of the motif ExxER/K located on the TMH1 in LjNPF4.6 (Fig. S1), reported also for other NPF proteins of the clade 4 (Longo et al., 2018), could be responsible of the observed uncoupling between proton binding and nitrate transport.

The achieved uptake of NO_3^- in *Xenopus* oocytes is confirmed *in planta* as the *Ljnpf4.6* mutants showed a 50% reduction of the $^{15}NO_3^-$ influx into roots compared to wild type, in 5 min short-uptake experiments (Fig. 4; 5 mM external nitrate). In *A. thaliana* two members of the NPF family have been mainly involved in the LATS, *AtNPF6.3* and *AtNPF4.6* with the latter being responsible for about 50% nitrate influx in short-term uptake measurements conducted the presence of 5 mM external nitrate (Huang et al., 1999). Therefore, the remaining NO_3^- uptake activity observed in the *Ljnpf4.6* background is likely due to the action of other *LjNPF* members. Furthermore, in *A. thaliana* a compensation effect mediated by NRT2 high-affinity transporters for loss of NO_3^- uptake has been reported (Ye et al., 2019).

The involvement of *LjNPF4.6* in the ABA-related signaling pathway controlling the *L. japonicus* root development is confirmed by the root phenotyping carried out in the presence of external ABA. The more striking effect of the ABA treatment we have scored in *L. japonicus* wild type plants was the inhibition of lateral root elongation already exhibited at the external concentrations of 0.1 and 1 μM ABA (Fig. S7B). The effect on primary root elongation was barely scored in that range of concentrations (Fig. S7A). The comparison of the root phenotypes displayed by wild type and *Ljnpf4.6* plants, clearly demonstrated the capacity of the mutants to counteract the ABA-dependent inhibitory effect on lateral root elongation, regardless of the use of Gln or KNO_3 as N sources (Fig. 7). A counteracting action of the mutants could not be scored for the primary root elongation phenotype in the presence of either 0.1 or 1 μM ABA (Fig. S9A) but this phenotype should be tested in the presence of a wider concentration of ABA (Fig. S7A). These phenotypic characterizations indicated a positive role played by *LjNPF4.6* in the root program response to exogenously applied ABA. A similar role in the ABA-related root elongation process has been recently reported for the *AtNPF4.6* gene, which shares with *LjNPF4.6* the capability to recognize and transport both nitrate and ABA substrates in *X. laevis* oocytes (Fig. 3; Huang et al., 1999; Léran et al., 2020). *Atnpf4.6* plants develop longer primary roots as compared to wild type plants, in the presence of 1 μM ABA (Zhang et al., 2021). The root phenotype of the *Atnpf4.6* plants was analyzed in young seedlings grown for 7 days with/out ABA 1 μM and lateral roots were not scored (Zhang et al., 2021). In wild type *Arabidopsis* plants, the ABA excess inhibits the phosphorylation of AtNPF4.6 by CEPR2, preventing its degradation via the 26S-mediated proteasome vacuolar pathway, hence allowing the maintenance of its import/export activity into the cell and transduction of the ABA signal excess with consequent inhibition of root growth (Zhang et al., 2021). However, some substantial differences can be found between the *AtNPF4.6*- and *LjNPF4.6*-related phenotypes. *AtNPF4.6* is mainly expressed in *A. thaliana* roots with a constitutive expression confined to the epidermis and root hairs of both young and mature roots before and after nitrate exposure (Huang et al., 1999). In contrast, *LjNPF4.6* is mainly expressed in *L. japonicus* leaves (Fig. 1A; Fig. S2) but displays a root specific nitrate-dependent profile of induction (Fig. 1B and C), with a promoter activity detected in different zones of transgenic hairy roots (Fig. 2).

The way nitrate and ABA may interact in the control of plant developmental programs is still a matter of debate. In the case of AtNPF4.6 a direct competition between nitrate and ABA substrates as the factor controlling the cross-talk between these two signals has been excluded because the ABA transport activity is not affected by an excess

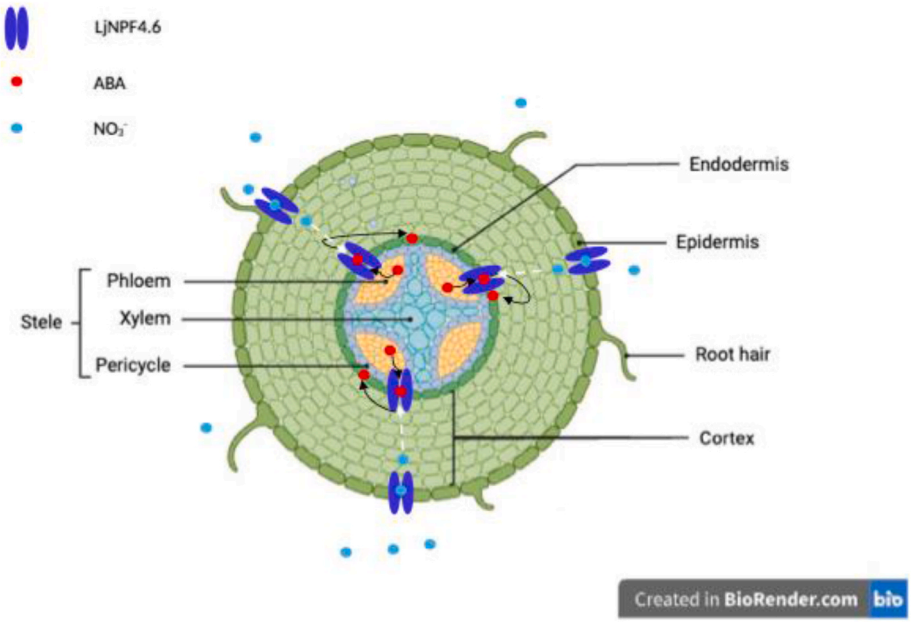

Fig. 9. Model of the NO_3^- responding pathway in *L. japonicus*. In the scheme proposed, *LjNPF4.6* plays a pivotal role in the signalling pathway (white arrows) linking the external nitrate signal with the lateral root elongation process controlled via the ABA accumulation in the endodermis of root tips. The curved black arrows indicate the export/import flux of ABA from the site of synthesis to the site of action. Created with BioRender, https://www.biorender.com/.

of nitrate and the tolerance of *Atnpf4.6* mutants to ABA-related inhibitory effect on germination is insensitive to the presence of nitrate (Kanno et al., 2013). A different mechanistic interaction between ABA and nitrate, which could explain the nitrate-related effects on root growth in *Arabidopsis* has been reported by Ondzighi-Assoume et al. (2016). The authors exploited an immunodetection approach to demonstrate the direct relationship between the presence of high exogenous nitrate (20–30 mM) and a peak of ABA accumulation in the *Arabidopsis* root tip endoderm that is functional to root elongation (Ondzighi-Assoume et al., 2016). The NO_3^--dependent ABA accumulation did not require ABA biosynthesis but rather depend by the release of bioactive ABA from the ABA-GE stored form, via the action of the ER-localized BG1 β-glucosidase. This mechanism is consistent with the SR phenotype exhibited by the *Atbg1* loss of function mutants that are also insensitive to the inhibitory effect of nitrate on root architecture (Ondzighi-Assoume et al., 2016). Interestingly in that work, the phenotypic characterization of *Atnpf4.6* mutants also indicated a defect in the NO_3^--dependent pattern of ABA accumulation, hence suggesting a contribute of ABA transport in that signaling pathway (Ondzighi-Assoume et al., 2016). The SR phenotype we observe in the *Ljnpf4.6* mutants resembles the one reported for the *Atbg1* and it is specifically displayed in the presence of nitrate as N source (Fig. 5). Therefore, we hypothesize that *LjNPF4.6* is involved in the root stele, in an import/export of ABA from synthesizing cells to target endodermis cells (Fig. 9). In the NO_3^-/ABA signaling pathway, the nitrate taken up within the root tissue via LjNPF4.6 could represent the signal controlling ABA transport through the post-translational mechanisms of regulation of ABA uptake and protein stability reported for AtNPF4.6 (Fig. 9; Zhang et al., 2021).

5. Conclusions

In conclusion, we report the positive role played by *LjNPF4.6* in the control of the *L. japonicus* root elongation program in response to NO_3^- and ABA stimuli. We propose that *LjNPF4.6* plays a pivotal role in the NO_3^-/ABA pathway, which controls the ABA distribution in the root endodermis in response to the external nitrate to promote root elongation. The mode of action of *LjNPF4.6* could be based on its dual capacity to respond to both nitrate and hormone signals in two physically separated zones of the root (Fig. 9). Further analyses of ABA distribution within the root tissues will be important to establish the mode of action of *LjNPF4.6*. Furthermore, the reported absence of effects of the *LjNPF4.6* mutation on the ABA-mediated nodulation phenotypes suggests a sharp separation between phytohormone dependent signaling pathways controlling root and nodule developmental processes.

CRediT authorship contribution statement

Ludovico M. Alves: Investigation, Methodology, Formal analysis, Review and editing. Vladimir T. Valkov: Investigation, Methodology, Formal analysis, Supervision, Data curation, Review and editing, Funding acquisition. Ylenia Vittozzi, Anita Ariante, Ani Barbulova, Thibaut Perez: Methodology, Investigation. Data curation. Alessandra Rogato: Methodology, Investigation, Data curation, Supervision. Benoit Lacombe: Resources, Conceptualization, Supervision, Review and editing. Maurizio Chiurazzi: Resources, Funding acquisition, Supervision, Conceptualization, Writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was financially supported by CNR project FOE-2019 DBA. AD003.139 and within the Agritech, National Research Center and received funding from the European Union Next Generation EU, Piano Nazionale di Ripresa e Resilienza (PNRR) – Missione 4, Componente 2, Investimento 1.4 - D.D.1032, June 17, 2022, CN00000022. This manuscript reflects only the author's views and opinions, neither the European Union nor the European Commission can be considered

responsible for them. This work was also financially supported by European Union-Next Generation and Italian Ministry of University and Research, (Progetti di Rilevanza Nazionale, PRIN 2022, CIRCLES Prot. 2022NW97JX. L.M.A. was supported by a Short-Term Fellowship from the European Molecular Biology Organization.

We thank Marco Petruzziello, Antonio Suppa and Giuseppina Zampi for technical assistance.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109144.

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